

Immunoreactivities to Rhodopsin and Rod/Cone Transducin Antisera in the Retina, Pineal Complex and Deep Brain of the Bullfrog, *Rana catesbeiana*

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ABSTRACT—Birds and lower vertebrates are known to have extra-retinal photoreceptors in the pineal complex and deep brain. Although the photoreceptive function of the pineal complex has been investigated well, the exact location and nature of the deep brain photoreceptors are not known. In this study we tried to localize visual pigments and signal transduction proteins immunohistochemically in the brain of bullfrogs (*Rana catesbeiana*). The retina, and the brain with the pineal and the frontal organ were fixed with Zamboni's fixative and/or Bouin's solution. Immunoreactivities to three antisera against bovine rhodopsin (Rh-As), α -subunits of bovine rod (anti-pTr α) and cone transducin (anti-pTc α) were shown in the retina, pineal, frontal organ and hypothalamus. The retina and pineal were immunopositive to both Rh-As and anti-pTr α , whereas the frontal organ was immunopositive to only Rh-As and the hypothalamus was immunopositive to all three antisera. The cells which were immunoreactive to Rh-As, anti-pTr α and anti-pTc α were observed in the preoptic nucleus and suprachiasmatic nucleus in the hypothalamus. The shape of these immunoreactive cells in the hypothalamus was round or spindle-like with one or two immunoreactive nerve processes most of which were perpendicular to the ventricular surface. Western blot analysis of the hypothalamus, pineal and frontal organ demonstrated immunoreactive bands molecular weight of which corresponded to those of the retina (34 kDa, 38 kDa and 41 kDa). Thus, visual pigments and transducin-like proteins seem to exist in the hypothalamus as well as the pineal complex of frogs.

INTRODUCTION

Birds and lower vertebrates are known to have extra-retinal photoreceptors, such as the pineal complex including the frontal organ in frogs and the parietal eye in lizards and deep brain photoreceptors. In the quail pineal, at least two types of photoreceptor cells are known [8, 14], and in the pineal and frontal organs of frogs, there are at least three types of photoreceptor cells [14, 20].

Since Benoit's pioneer works [1], many studies on the deep brain photoreceptors have been done in avian species. The photoreceptors which mediate photoperiodism may well lie in the medio-basal hypothalamus [10, 18] according to a local illumination experiment established by Oishi and Kato [15]. Action spectra for the photoperiodic responses suggested the involvement of rhodopsin-like photopigment in the hypothalamus in intact quail [5, 6] and in blinded and pinealectomized quail [16]. In immunohistochemical studies, Silver *et al.* [19] reported that there were cells labeled with an opsin antibody in two distinct regions, the lateral septum and the infundibular region of the hypothalamus. But they failed to detect opsin in immunoblot analysis. Although Oishi, Yoshikawa and Yashiro (unpublished data) also showed positive results, Foster *et al.* [7] could not detect any immunopositive cells to opsin antibodies in the quail

hypothalamus.

The cells at the ventricular border of septum in lizards were labeled with opsin antibodies, and opsins and retinals were detected in the anterior brain by immunoblot analysis and high performance liquid chromatography analysis, respectively [9].

In frogs, responses to white light stimulation can be recorded electrophysiologically from diencephalon and mesencephalon [2, 3]. Chromophores of rhodopsin, 11-cis and all-trans retinal, were also detected in the ventral part of brain including the hypothalamus [14].

Since antibodies against α -subunits of bovine rod and cone transducins (Tr α and Tc α , respectively) have been developed and shown to react to rod and cone outer segments, respectively, in bovine [13] and chicken retinas [12], these antisera can classify the rod type and cone type photoreceptors and signal transductions. In this study, we tried to reveal the existence and localization of rhodopsin and transducins in the retina, pineal complex and deep brain in the bullfrog by means of immunohistochemistry and immunoblot analysis.

MATERIALS AND METHODS

Experimental animals

Adult bullfrogs (*Rana catesbeiana*) were obtained from a local breeder.

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Immunohistochemistry

The eyes and brains with the pineal and frontal organ were dissected ($N=13$). The tissues were fixed with Zamboni's fixative and/or Bouin's solution. After the tissues were dehydrated through graded alcohols, they were embedded in paraffin and sectioned at 4–6 μm . Deparaffinized sections were washed in PBS and immersed in 0.3% H_2O_2 in methanol to remove endogenous peroxidase.

As described previously [17], sections thus prepared were treated with ABC (avidin-biotin peroxidase complex) method. Briefly, after PBS wash, the sections were incubated with diluted normal serum (30 min), with primary antisera (over night at 4°C), with biotinylated antibody solution (60 min), and with Vectastain ABC reagent (Vector, Burlingame) (30 min). Then peroxidase activities were demonstrated by diaminobenzidine (DAB; Nacalai Tesque, Kyoto) solution. The sections were washed in PBS for 30 min between each incubation. Normal serum, primary antisera, biotinylated antibody and ABC reagent were diluted with 0.3% triton X-100 in PBS. All processes except the incubation with primary antisera described above were done at room temperature. Anti-bovine rhodopsin antiserum (Rh-As, dilution: $\times 1000$ or $\times 500$ as described by Kawata *et al.* [11]), and antisera against synthetic oligopeptides [13] specific to α -subunits of bovine rod and cone transducins (anti-pTra and anti-pTca, respectively, dilution: $\times 500$ as described by Kokame *et al.* [12]) were used as the primary antisera.

The preparations were observed with a Nomarski differential interference microscope.

Western blotting

The retina, hypothalamus, pineal, frontal organ, hypophysis and cerebrum were dissected out from six frogs and frozen in dry-ice alcohol and kept in a deep freezer (-85°C) until use. Each tissue except the retina was homogenized in 1% digitonin in PBS, and the retina was homogenized in saline. The homogenates were centrifuged (15000 rpm for 20 min), and each pellet was homogenized in 1% digitonin in PBS and centrifuged again. The supernatant was used for western blotting. Protein extracts were applied to 12% polyacrylamide gel and electrophoretically separated. Proteins in the gel were transferred to a Poly Screen sheet (Du Pont, Boston), and then, treated with the ABC method. Following processes, except the incubation with primary antisera were done at room temperature. The sheet of Poly Screen was incubated with 1% skim milk in PBS for 1 hour to block non-specific reaction, and then primary antiserum (over night at 4°C) and biotinylated secondary antibody (1 hour) diluted with 1% skim milk in PBS. Poly Screen sheet was washed with 1% skim milk in PBS between these incubations. Then the sheet was washed with 0.05% Triton X-100 in PBS to remove skim milk, and incubated with Vectastain ABC reagent. DAB solution or western blot chemiluminescence reagent (Du Pont, Boston) was used to visualize immunoreactive bands.

RESULTS

Immunohistochemistry

The outer segments and some parts of inner segments of rods in the retina were immunopositive to Rh-As (Table 1)

TABLE 1. Results of immunohistochemistry in the retinal and extra-retinal photoreceptors of bullfrogs

		Retina	Pineal	Frontal organ	Hypothalamus
Rh-As	B	++	++	++	++
	Z	++	++		—
	Z+B	++	++		+
anti-pTra	B	—	—	—	++
	Z	++	—		—
	Z+B	++	+	—	+
anti-pTca	B	—	—	—	++
	Z	—	—		—
	Z+B	—	—	—	+

Tissues were fixed with Bouin's solution (B), Zamboni's fixative (Z) or a combination of Zamboni's fixative and Bouin's solution (Z+B).

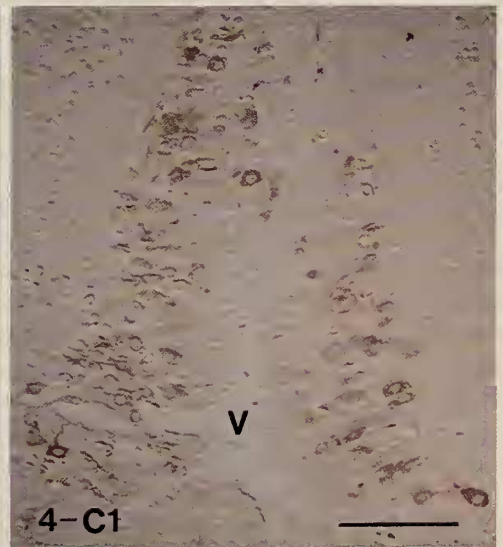
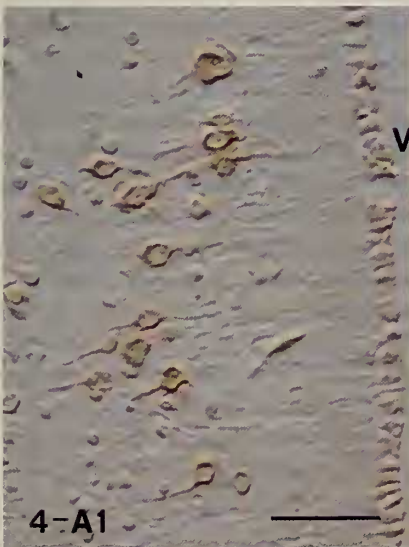
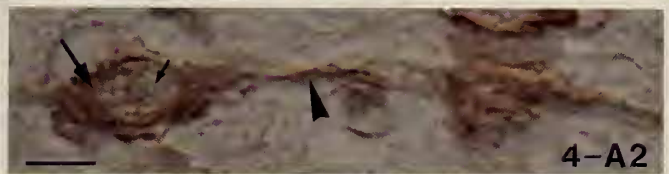
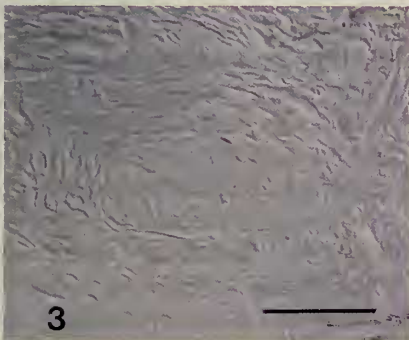
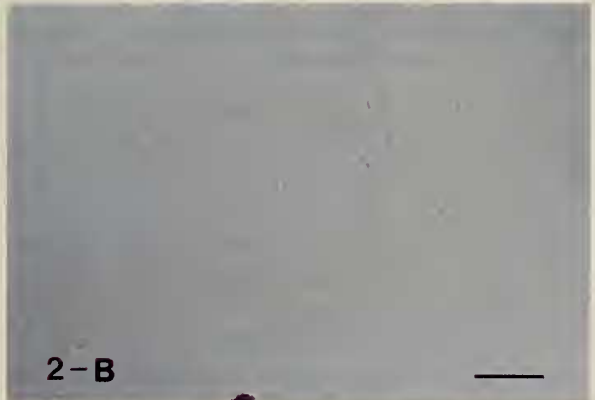
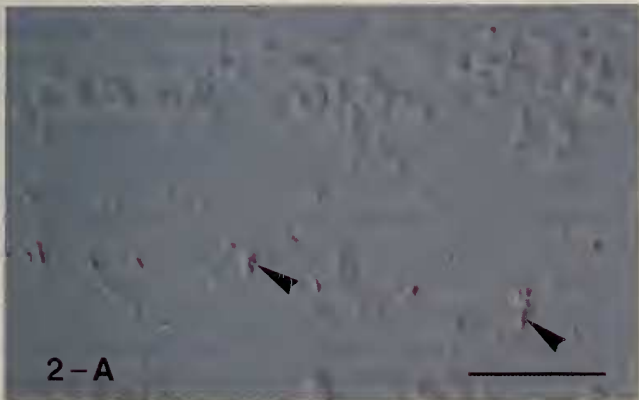
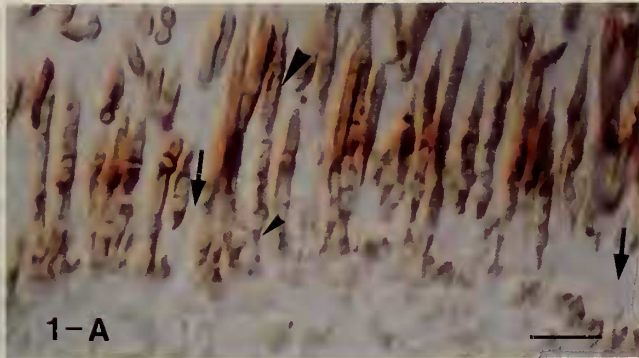
+: immunopositive, —: immunonegative

FIG. 1. Immunohistochemistry of the photoreceptor cells in the retina fixed with Zamboni's fixative. Anti-pTra labeled rod outer ($\blacktriangleright$) and inner segment ($\blacktriangleright$) (A), but did not label cone cells ($\rightarrow$). There were no immunoreactive cells to anti-pTca (B). Scale bar=20 μm .

FIG. 2. Immunohistochemistry of the pineal fixed with Zamboni's fixative and Bouin's solution. The outer segments of the pinealocytes were stained with anti-pTra ($\blacktriangleright$) (A). There were no immunoreactivities to anti-pTca (B). Scale bar=40 μm .

FIG. 3. Immunohistochemistry of the frontal organ fixed by Zamboni's fixative and Bouin's solution. There were no immunoreactivities to anti-pTca. Scale bar=100 μm .

FIG. 4. Immunohistochemistry in the hypothalamus fixed with Bouin's solution. Three antisera were used; Rh-As (A), anti-pTra (B) and pTca (C). Stained cells were located near the third ventricle (V). There were no differences in the extent of immunoreactivity and distribution of immunoreactive cells among each antiserum. The immunoreactive cells distributed in the suprachiasmatic nucleus (C1) and preoptic nucleus (A1, B1). The cells showed round or spindle-like shape and had immunoreactive nerve processes ($\blacktriangleright$), most of which were perpendicular to the ventricular surface in cross sections. These cells have immunopositive cytoplasm ($\rightarrow$) and immunonegative nucleus ($\rightarrow$) (A2, B2 and C2). The diameter of these cells was 9.7–21.5 μm , and the average was 15.0 μm . A1, B1, C1: Scale bar=100 μm . A2, B2, C2: Scale bar=20 μm .



and anti-pTr α (Fig. 1-A), while there were no immunoreactivity to anti-pTc α (Fig. 1-B). But there were some differences in the immunoreactivities to anti-pTr α depending on the fixative used (Table 1), i.e., the result was negative when Bouin's solution was used. Cone cells were immunonegative to all three antisera.

The outer segments and cell bodies of many pinealocytes were immunopositive to Rh-As (Table 1). The outer segments of some pinealocytes were immunopositive to anti-pTr α (Fig. 2-A), while the other cells were immunonegative to the antiserum. But the positive immunoreactivities to anti-pTr α were obtained only in the pineal fixed with a combination of Zamboni's fixative and Bouin's solution, showing difference depending on the fixative used (Table 1). There were no cells immunopositive to anti-pTc α (Fig. 2-B).

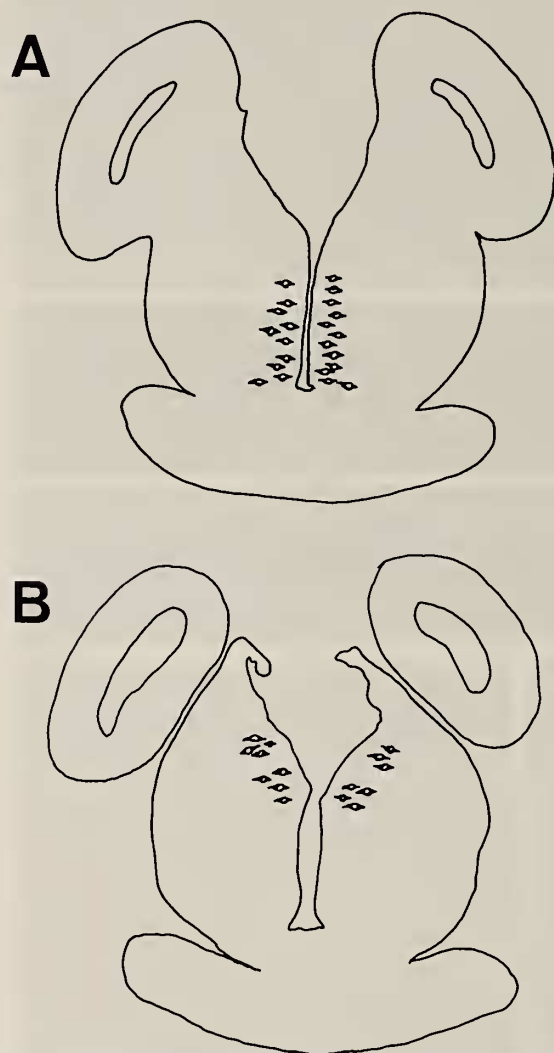


FIG. 5. Schematic drawing of cross sections of the brain. The areas where immunoreactive cells located were shown (A, B). These areas corresponded to the suprachiasmatic nucleus (A) and preoptic nucleus (B) in the hypothalamus. The immunoreactive cells in the suprachiasmatic nucleus were located in front of the preoptic nucleus. Population density of these cells was higher in the suprachiasmatic nucleus than in the preoptic nucleus.

The frontal organ was immunopositive to Rh-As but immunonegative to both anti-pTr α and anti-pTc α (Fig. 3, Table 1).

The cells in the hypothalamus near the third ventricle were immunopositive to all three antisera (Rh-As, anti-pTr α and anti-pTc α) (Fig. 4-A, B, C). The area where the immunopositive cells were observed was divided into two parts (Fig. 5), and these areas corresponded to the suprachiasmatic nucleus and preoptic nucleus. There were the pineal and choroid plexus in the upper part of third ventricle of the area. The immunoreactive cells in the suprachiasmatic nucleus were located in front of the preoptic nucleus. Population density of immunopositive cells was higher in the suprachiasmatic nucleus (Fig. 4-C1) than in the preoptic nucleus (Fig. 4-A1, B1). The results obtained from serial sections suggest that all three immunoreactive substances were localized in one and the same cells. The shape of immunoreactive cells was round or spindle-like with one or two immunoreactive nerve processes, most of which were perpendicular to the ventricular surface in cross sections (Fig. 4-A1, C1). In sagittal sections, immunoreactive nerve processes were not observed, indicating that the direction of nerve processes is perpendicular to the sagittal axis. There were no differences in the extent of immunoreactivity among each antiserum. No particular structures except the nucleus were observed in the immunoreactive cells (Fig. 4-A2, B2, C2). All these cells had immunopositive cytoplasm and immunonegative nucleus. The diameter of these cells ranged from 9.7 μ m to 21.5 μ m, and the average was 15.0 μ m. In the hypothalamus, there were some differences in immunoreactivities among the fixatives used (Table 1), i.e., the immunoreactivities to all three antisera became unclear when Zamboni's fixative was used.

Positive immunoreactivities to Rh-As and anti-pTr α were observed in the retina, pineal and hypothalamus, but the immunoreactivity to anti-pTc α was positive only in the hypothalamus (Table 1).

Western blot analysis

Immunoblot analysis of retinal extracts with Rh-As demonstrated two bands, molecular weights of which were 34 kDa and 36 kDa, and the analysis with anti-pTr α and anti-pTc α demonstrated a band of 41 kDa and 38 kDa, respectively (Fig. 6). In the extracts of the hypothalamus, pineal and frontal organs, 34 kDa band was demonstrated with Rh-As (Fig. 6). There were immunoreactive bands to anti-pTc α in the extracts of the hypothalamus and cerebrum, molecular weights of which were 38 kDa and 120~130 kDa (Fig. 6). Some bands except those described above were also demonstrated by immunoblot analysis, but they were non-specific bands to the antisera, because those bands were demonstrated without the primary antisera.

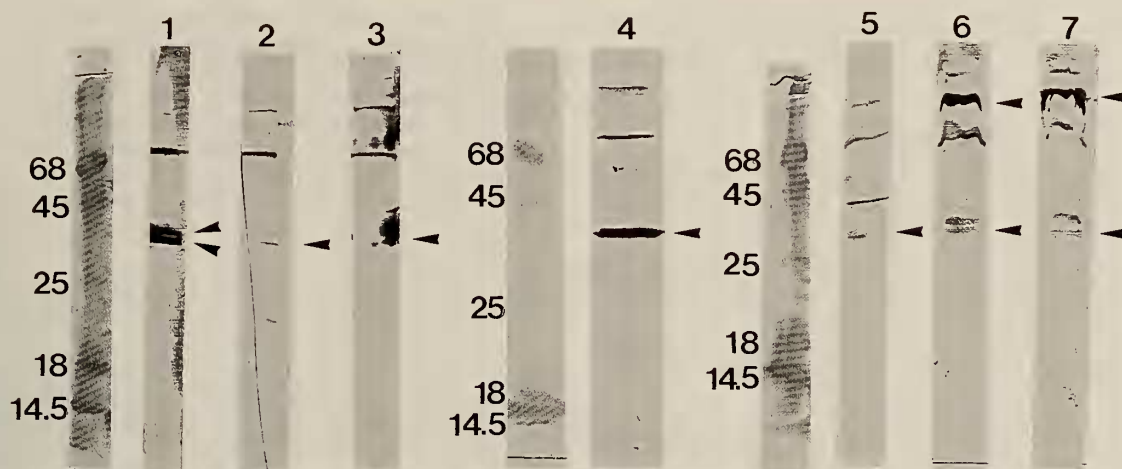


FIG. 6. Immunoblot analysis of protein extracts from tissues. Immunoreactive bands were indicated by arrow heads (►). Immunoreactive bands to Rh-As were detected in the extracts of retina (lane 1) (34 kDa, 36 kDa), pineal (lane 2) and hypothalamus (lane 3) (34 kDa). An immunopositive band to anti-pTr α was detected in the retinal extract (lane 4) (41 kDa), and those to anti-pT α were detected in the extracts of retina (lane 5) (38 kDa), hypothalamus (lane 6) and cerebrum (lane 7) (38 kDa, 120~130 kDa).

DISCUSSION

The present study using Rh-As in the retina and pineal complex confirmed our previous results [11, 14]. Since the pineal was immunopositive to antibodies against Rh and pTr α (Fig. 2), the pineal might also have rod-like photoreceptors and a signal transduction system. Since the frontal organ was immunoreactive to Rh-As but not to anti-pTr α and -pT α (Fig. 3), the signal transduction system in the frontal organ might be different from the retina or transducin molecules might have sequences different from bovine transducins, and can not be identified by the antibodies used. There were some immunoreactive cells to the antisera against photoreceptor proteins (rhodopsin and transducins) in the supra-chiasmatic nucleus and preoptic nucleus of the hypothalamus (Fig. 4). Immunoblot analysis of the hypothalamus with Rh-As demonstrated an immunoreactive band and the molecular weight corresponded to 34 kDa band of the retina (Fig. 6). Masuda *et al.* [14] detected 11-cis and all-trans retinal in the ventral part of the frog diencephalon including the hypothalamus. These results indicate that visual pigments, chromophores and proteins involved in the signal transduction of light exist in the hypothalamus of frogs. The mesencephalic and diencephalic unit of the blinded and pinealectomized frogs showed electrophysiological responses to light [2, 3]. These responses might be induced by photoreception through rhodopsin system. From the immunohistochemical results of serial sections, we suppose these immunoreactive substances to the three antisera are localized together in the same cells in the hypothalamus.

When the retinas were fixed with Bouin's solution, the outer segments of photoreceptor cells were immunopositive to Rh-As and immunonegative to anti-pTr α . However, they were immunopositive to both Rh-As and anti-pTr α when Zamboni's fixative or a combination of Zamboni's fixative and Bouin's solution was used. The same results were

obtained from the retina of Japanese quail (*Coturnix coturnix japonica*, unpublished data). Since rhodopsin is bound to disk membranes and transducins are peripheral proteins, differences in the immunoreactivity depending on the fixatives and tissues might be due to differences in characters of proteins. When the hypothalamus was fixed with Bouin's solution, there was an intense immunoreactivity to all these antisera (Fig. 4), while there were no immunoreactivities when it was fixed with Zamboni's fixative. When fixed with Zamboni's fixative and then Bouin's solution, there was a weak immunoreactivity (Table 1). Thus, the immunoreactivity of the hypothalamus to the antisera was different from the retina depending on each fixative used. Since we do not know how photoreceptor proteins are localized in the cells of the hypothalamus, immunohistochemistry using an electron microscope is now in progress in our laboratory.

Immunoblot analysis with Rh-As and anti-pTr α in retinal extracts clearly demonstrated immunoreactive bands. The result with Rh-As corresponded to those reported by Fong *et al.* [4]. Other bands showed very weak immunoreactivities, though they were specific to each antiserum.

Locomotor activity of blinded frogs (*Xenopus*) can be entrained to light-dark cycles (Fujisawa, Harada, Kegasawa and Oishi, in preparation). Since the pineal of *Xenopus* is not developed well, the results indicate that the deep brain photoreceptor (probably hypothalamus) is involved in the entrainment of circadian rhythms as the extra-retinal and extra-pineal photoreceptor. Our results suggest that the visual pigments involved in these phenomena are rhodopsin-like substances and that the signal transduction is mediated by transducin-like substances.

Foster *et al.* [9] reported opsin localization and existence of retinoids in the brain of lizards. There were immunopositive cells to a monoclonal antibody against opsin in the brain of ring doves [19]. These results in addition to our present results indicate that rhodopsin-like photopigments involved

in the brain photoreceptor prevail among birds and lower vertebrates.

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